Research Paper

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Wnt/Ryk signaling contributes to neuropathic pain by regulating sensory neuron excitability and spinal synaptic plasticity in rats

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Abstract

Treating neuropathic pain continues to be a major clinical challenge and underlying mechanisms of neuropathic pain remain elusive. We have recently demonstrated that Wnt signaling, which is important in developmental processes of the nervous systems, plays critical roles in the development of neuropathic pain through the β-catenin–dependent pathway in the spinal cord and the β-catenin–independent pathway in primary sensory neurons after nerve injury. Here, we report that Wnt signaling may contribute to neuropathic pain through the atypical Wnt/Ryk signaling pathway in rats. Sciatic nerve injury causes a rapid-onset and long-lasting expression of Wnt3a, Wnt5b, and Ryk receptors in primary sensory neurons, and dorsal horn neurons and astrocytes. Spinal blocking of the Wnt/Ryk receptor signaling inhibits the induction and persistence of neuropathic pain without affecting normal pain sensitivity and locomotor activity. Blocking activation of the Ryk receptor with anti-Ryk antibody, in vivo or in vitro, greatly suppresses nerve injury-induced increased intracellular Ca^{2+} and hyperexcitability of the sensory neurons, and also the enhanced plasticity of synapses between afferent C-fibers and the dorsal horn neurons, and activation of the NR2B receptor and the subsequent Ca^{2+}-dependent signals CaMKII, Src, ERK, PKCγ, and CREB in sensory neurons and the spinal cord. These findings indicate a critical mechanism underlying the pathogenesis of neuropathic pain and suggest that targeting the Wnt/Ryk signaling may be an effective approach for treating neuropathic pain.

Keywords: Nerve injury, Wnt, Ryk, Neural excitability, Synaptic plasticity

1. Introduction

Treatment of neuropathic pain, which is caused by nerve injury or other forms of stress or disease in the nervous system, continues to be a major clinical challenge. Despite decades of investigation and numerous implicated processes, the specific cellular and molecular mechanisms underlying neuropathic pain remain elusive, and clinical approaches for treating neuropathic pain are limited. We have recently found that Wnt signaling is involved in the development of neuropathic pain after nerve injury in the dorsal root ganglion (DRG) and the spinal cord levels. Activated Wnt signaling pathways play important roles in induction and persistence of neuropathic pain.[35] Wnt signaling may be a critical mechanism underlying the pathogenesis of neuropathic pain.[10,12] Wnts are a family of secreted lipid-modified signaling proteins acting as short- or long-range signaling molecules in regulating cellular processes during the development of nervous systems[5,24] and in regulating synaptic plasticity.[2,4,9] Wnt ligands bind to the cysteine-rich domain of Frizzled receptors and the coreceptors to activate intracellular signaling cascades. Typical Wnt signaling pathways include the canonical Wnt/β-catenin pathway and noncanonical β-catenin–independent pathways.[4,5,13,27,34] Our findings have demonstrated that activation of Wnt signaling is involved in the development of neuropathic pain by stimulating production of proinflammatory cytokines interleukin-18 (IL-18) and tumor necrosis factor-α (TNF-α) through the β-catenin–dependent pathway in the spinal cord and through the β-catenin–independent pathway in DRG neurons.[35] In addition, Wnts may function through an atypical Wnt/Ryk signaling pathway to activate intracellular signaling cascades.[7,25,38] Ryk is a single span transmembrane receptor with an intracellular tyrosine kinase domain and a recently uncovered atypical receptor in Wnt signaling. The Ryk receptor is required for various Wnt signaling functions involved in many diverse roles in the developing nervous system.[11,18,26] In this study, we investigated roles of Wnt/Ryk signaling in the pathophysiology of neuropathic pain using well-characterized rat models of chronic constriction injury (CCI) of the sciatic nerves[1] and nociceptive C-afferent fibers-mediated enhancement of synaptic plasticity of the dorsal horn neurons.[15,16,36] The results showed that CCI increases expression of Wnt3a, Wnt5b, and Ryk (the transmembrane and the intracellular domains) in the DRG and the spinal cord. Spinal blocking of Wnt/Ryk signaling inhibits the induction and maintenance of mechanical allodynia and thermal hyperalgesia after CCI. Ryk-mediated Wnt signaling may contribute to the mechanisms underlying neuropathic pain

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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by regulating the excitability of DRG neurons and nociceptive synaptic transmission in the dorsal horn through modulating intracellular Ca^{2+}, the activation of the NR2B receptor, and subsequent Ca^{2+}-dependent signals.

2. Materials

2.1. Animals, anesthesia, drugs, and drug administration

Adult male Sprague–Dawley rats (200-220 g-wt) (Peking University Animal Center, Beijing, China) were used. Our experimental procedures and animal use were conducted in accordance with the regulation of the ethics committee of the International Association for the Study of Pain and approved by the Animal Care and Use Committee at Peking University Health Science Center. All surgeries were performed under anesthesia with sodium pentobarbital intraperitoneally (50 mg/kg, i.p.). We purchased Wnt agonist named "Wnt agonist," an exogenous, selective activator of Wnt signaling, from EMD Chemicals Inc (Philadelphia, PA); anti-Ryk antibody (anti-Ryk), functional-blocking Ryk receptor, and control IgG from R&D Systems (Minneapolis, MN); N-methyl-D-aspartate (NMDA) receptor agonist NMDA and NMDA receptor antagonist dizocilpine (MK-801) from Sigma-Aldrich Co (St. Louis, MO). The drugs were delivered intrathecally (i.t., in volume of 20 μL) into cerebral spinal fluid through lumbar puncture.

2.2. Chronic constriction injury

To produce peripheral nerve injury, a rat model of CCI was used in this study. Under anesthesia, the left common sciatic nerve of each rat was exposed at the mid-thigh level. Proximal to the sciatic nerve's trifurcation was freed of adhering tissue and 4 ligatures (4-0 surgical catgut) were tied loosely around it with approximately 1 mm between ligatures. Animals in the sham group received surgery identical to that described in CCI but without nerve injury.

2.3. Assessment of mechanical allodynia and thermal hyperalgesia

Mechanical allodynia was determined by measuring incidence of foot withdrawal in response to mechanical indentation of the plantar surface of each hind paw with a sharp, cylindrical probe with a uniform tip diameter of approximately 0.2 mm provided by an Electro Von Frey (ALMEMO 2390-5 Anesthesiometer; IITC Life Science Inc, Woodland Hills, CA). The probe was applied to 6 designated loci distributed over the plantar surface of the foot. The minimal force (in grams) that induced paw withdrawal was read off the display. Threshold of mechanical withdrawal in each animal was calculated by averaging the 6 readings and the force was converted into milli-newtons (mN). Thermal hyperalgesia was assessed by measuring foot withdrawal latency to heat stimulation. An analgesia meter (IITC Model 336 Analgesia Meter, Series 8; IITC Life Science Inc) was used to provide a heat source. In brief, each animal was placed in a box containing a smooth, temperature-controlled glass floor. The heat source was focused on a portion of the hind paw, which was flush against the glass, and a radiant thermal stimulus was delivered to that site. The stimulus shut off when the hind paw moved (or after 20 seconds to prevent tissue damage). The intensity of the heat stimulus was maintained constant throughout all experiments. The elicited paw movement occurred at latency between 9 and 15 seconds in control animals. Thermal stimuli were delivered 3 times to each hind paw at 5- to 6-minute intervals. For the results expressing mechanical allodynia or thermal hyperalgesia, the values are mean of ipsilateral feet. These protocols used for determining the pain-related behaviors were similar to those we have previously described.[14,29–31]

2.4. Quantitative real-time polymerase chain reaction

Under deep anesthesia, the L4-L5 spinal cord segments of rats were quickly removed and analyzed. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA was then synthesized using the Thermo Scientific Verso cDNA synthesis kit (Thermo Scientific, ABgene, Surrey, United Kingdom) with oligo(dT)18 primer. Quantitative real-time polymerase chain reaction was performed with DyNAmo Flash SYBR Green qPCR kit (ThermoFisher Scientific, Waltham, MA). The thermal cycle conditions used for the detection of the Ryk gene was 95°C for 7 minutes, 45 cycles of 95°C for 10 seconds and 58°C for 45 seconds. Specific primers used for the detection of Ryk sequence were as follows. Gene Ryk: forward (5′-3′)—TTCCGATGGACTACCACTGT; reverse (5′-3′)—GGGCTACGGTAACCATCT; accession#—NM_080,402. Gene GAPDH: forward (5′-3′)—AGACAGCCGCATCTTCTTGT; reverse (5′-3′)—CTTGCCGTGGGTAGAGTCAT; accession#—NM_017,008. Relative mRNA levels were calculated using the $2-\Delta\Delta CT$ method. Gene expression was first normalized to the housekeeping control gene GAPDH, and subsequently, the relative expression of genes of interest was compared with the respective experimental control.

2.5. Protein determinations

To quantify temporal changes in protein levels of the Wnts and Ryk signals, Western blotting analysis was used. The L_4-L_5 DRG and/or spinal cord segments were quickly removed from deeply anesthetized rats and stored at −80°C. Sequential precipitation procedures were used on the tissue samples that were lysed in ice-cold (4°C) NP-40 or RIPA lysis buffer containing a cocktail of protease inhibitor, phosphatise inhibitors, and phenylmethylsulfonyl fluoride (Sigma-Aldrich). The protein concentrations of the lysates were estimated using the method of BCA (with reagents from Pierce, Rockford, IL) and the total protein content between samples was equalized. The total protein was separated by SDS-PAGE and transferred to 0.2 μm nitrocellulose or PVDF membrane (both from Bio-Rad Laboratories, Hercules, CA). The following primary antibodies were used: anti-Wnt3a (1:1000, Millipore Co, Billerica, MA), anti-Wnt5b (1:1000, Abcam, Cambridge, MA), anti-Ryk (1:800, 4°C overnight, Thermo Scientific Co, Rockford, IL), anti-pNR2B (Tyr^{1472}) (1:300, Millipore), anti-pSrc (pY^{418}) (1:1000, Biosource, Camarilo, CA), anti-pCaMKII (Thr^{286}, 3361) (1:1000, Cell Signaling Tech, Beverly, MA), anti–pPKC-γ (Thr^{514}, 1:1000, 4°C overnight), anti-pCREB (Ser^{133}) (Cell Signaling Technology, Inc, Danvers, MA), and anti-GAPDH (1:5000∼35,000, Sigma-Aldrich). The membranes were then developed by enhanced chemiluminescence reagents (Perkin Elmer, Waltham, MA) with horseradish peroxidase–conjugated secondary antibodies (R&D System). Data were analyzed with the Molecular Imager (ChemiDoc XRS, Bio-Rad Laboratories) and the associated software Quantity One-4.6.5 (Bio-Rad Laboratories).

2.6. Immunohistochemistry

Deeply anesthetized rats were perfused transcardiacally with 0.9% saline and followed by 4% formaldehyde. The L_4-L_5 spinal cord segments and DRG were removed and postfixed in 4% formaldehyde overnight. Sections (40 μm) were cut using

a vibratome (Vibratome 1000 Plus; Vibratome Co, St. Louis, MO). For immunofluorescence staining, free-floating sections were blocked in TBS contain 5% donkey serum or 5% goat serum according to host specie of secondary antibody for 2 hours and incubated in first primary antibody at 4°C overnight. Sections were then washed in 0.05M Tris-HCl (pH 7.4) (3 × 5 minutes) and followed by incubating in the secondary antibody at room temperature for 2 hours and washing. For double staining, the same procedure was performed to the second primary and secondary antibodies. Sections were mounted on slides and covered with 90% glycerin for observation under a confocal microscope (FluoView FV1000, Olympus Co, Tokyo, Japan). The dilution of antibodies used included anti-Ryk (1:100, Abgent, San Diego, CA), anti-NeuN (1:100∼200, Millipore, Temecula, CA), anti-GFAP and anti-NF200 (both with 1:100, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-Iba1 and anti-CGRP (1:400 and 1:200, Abcam), and anti-IB4 (1:100, Sigma-Aldrich). The anti-Ryk antibody was used for the first time in our laboratory for this study, and we performed the antibody absorption controls in the DRG and the spinal cord and confirmed that the anti-Ryk antibody was highly specific.

2.7. Excised, intact in vitro ganglion preparation

This preparation allows us to test DRG neurons while still in place in excised ganglia. The protocol was the same as that we have described previously.[30,32,37] Under deep anesthesia, a laminectomy was performed, and the L4 and/or L5 DRG with attached sciatic nerve and the dorsal roots were removed and placed in 35-mm petri dishes containing ice-cold oxygenated ACSF consisting of (in mM) 140 NaCl, 3.5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 4.5 HEPES, 5.5 HEPES-Na, and 10 glucose (pH 7.3). The perineurium and epineurium were peeled off, and the attached sciatic nerve and dorsal roots were transected adjacent to the ganglion. The intact ganglion was treated with collagenase (type P, 1 mg/mL, Roche Diagnostics, Indianapolis, IN) for 30 minutes at 35°C and then incubated at room temperature for patch-clamp recordings or $[Ca^{2+}]_i$ measurement.

2.8. Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Intact ganglions were prepared and incubated in ACSF containing Fura-2/AM (5 μM) and Pluronic F-127 (0.5 mg/mL) (Invitrogen). Fluorescence in the small and medium-sized DRG neurons (diameter, 15–45 μm), but not the glial cells surrounded, was measured at 340 and 380-nm excitation and 520-nm emission (Olympus IX51 with ORCA-R2 digital camera, Hamamatsu Inc, Japan). The 340/380-nm emission ratio was used to determine $[Ca^{2+}]_i$. After each recording, 4-bromo A-23187 (BR-A, 10 μM, Sigma) was used to check the viability of the cells.

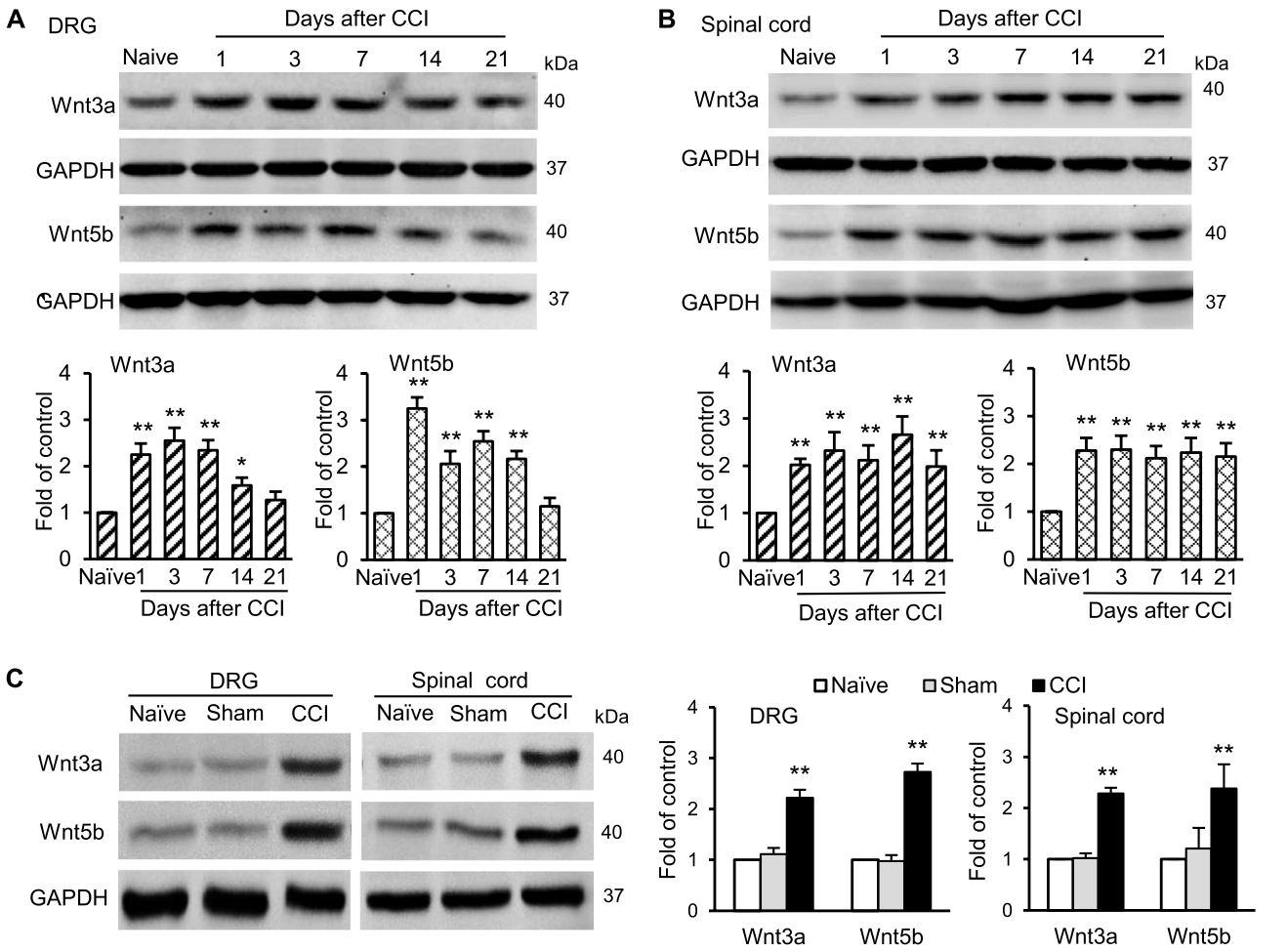

Figure 1. Expression of Wnt3a and Wnt5b protein in dorsal root ganglion (DRG) and the spinal cord after chronic constriction injury (CCI). Western blotting showing time course of expression of Wnt3a and Wnt5b in DRG (A) and the spinal cord (B) after CCI. (C) Comparison of expression of Wnt3a and Wnt5b in DRG and the spinal cord in naive (day 0), Sham (day 7), and CCI (day 7) rats. Top: representative bands; bottom: data summary. Data are expressed as mean ± SEM (n = 4 in each group in A and B; n = 3 in each group of C). *$P < 0.05$, **$P < 0.01$ vs naive in the corresponding group (1-way analysis of variance).

2.9. Whole-cell current-clamp recordings

To test excitability of the nociceptive DRG neurons, whole-cell current-clamp and voltage-clamp recordings were made with an Axopatch-200B amplifier (Molecular Devices, Union city, CA) in the small DRG neurons (soma diameter: 15-30 μm) from the intact ganglion preparations. These neurons largely correspond to neurons with C-fiber conduction velocities. Conduction velocity was not measured in this study. The protocols were similar to that we have previously described.[32,37] Glass electrodes were fabricated with a Flaming/Brown micropipette puller (P-97, Sutter instruments, Novato, CA). Electrode impedance was 3 to 5 MΩ when filled with saline containing (in mM) 120 K^+-gluconate, 20 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 ethylene glycol-bis-(β-aminoethyl-ether) N,N,N′,N′,-tetraacetic acid, 2 Mg-ATP, and 10 HEPES-K (pH 7.2, osmolarity 290-300 mOsm). Electrode position was controlled by a 3-D hydraulic micromanipulator (MHW-3, Narishige). When the electrode tip touched the cell membrane, gentle suction was applied to form a tight seal (serial resistance >2GΩ). Under −70 mV command voltage, additional suction was applied to rupture the cell membrane. After obtaining the whole-cell mode, the recording was switched to current-clamping mode and the resting membrane potential (RMP) was recorded.

All the DRG cells accepted for analysis had an RMP of −45 mV or more negative. To compare the excitability of the DRG neurons, we examined the RMP, action potential current threshold (APCT), and repetitive discharges evoked by a standardized intracellular depolarizing current. The RMP was taken 2 to 3 minutes after a stable recording was first obtained. Action potential current threshold was defined as the minimum current required evoking an action potential by delivering intracellular currents from −0.1 to 0.7 nA (50 milliseconds pulses) in increments of 0.05 nA. The whole-cell input capacitance (C_{in}) was calculated by integration of the capacity transient evoked by a 10 mV pulse in voltage clamp mode. Repetitive discharges were measured by counting the spikes evoked by 1000 milliseconds, intracellular pulses of depolarizing current normalized to 2.5 times APCT. All electrophysiological recordings and data analyses were conducted by experimenters blind to previous treatment of the cells.

2.10. Extracellular recordings of C-fiber-evoked field potential and long-term potentiation of synapses between C-fibers and the dorsal horn neurons in vivo

Under anesthesia, a cannula was inserted into the rat's trachea to allow artificial ventilation and another cannula containing heparin

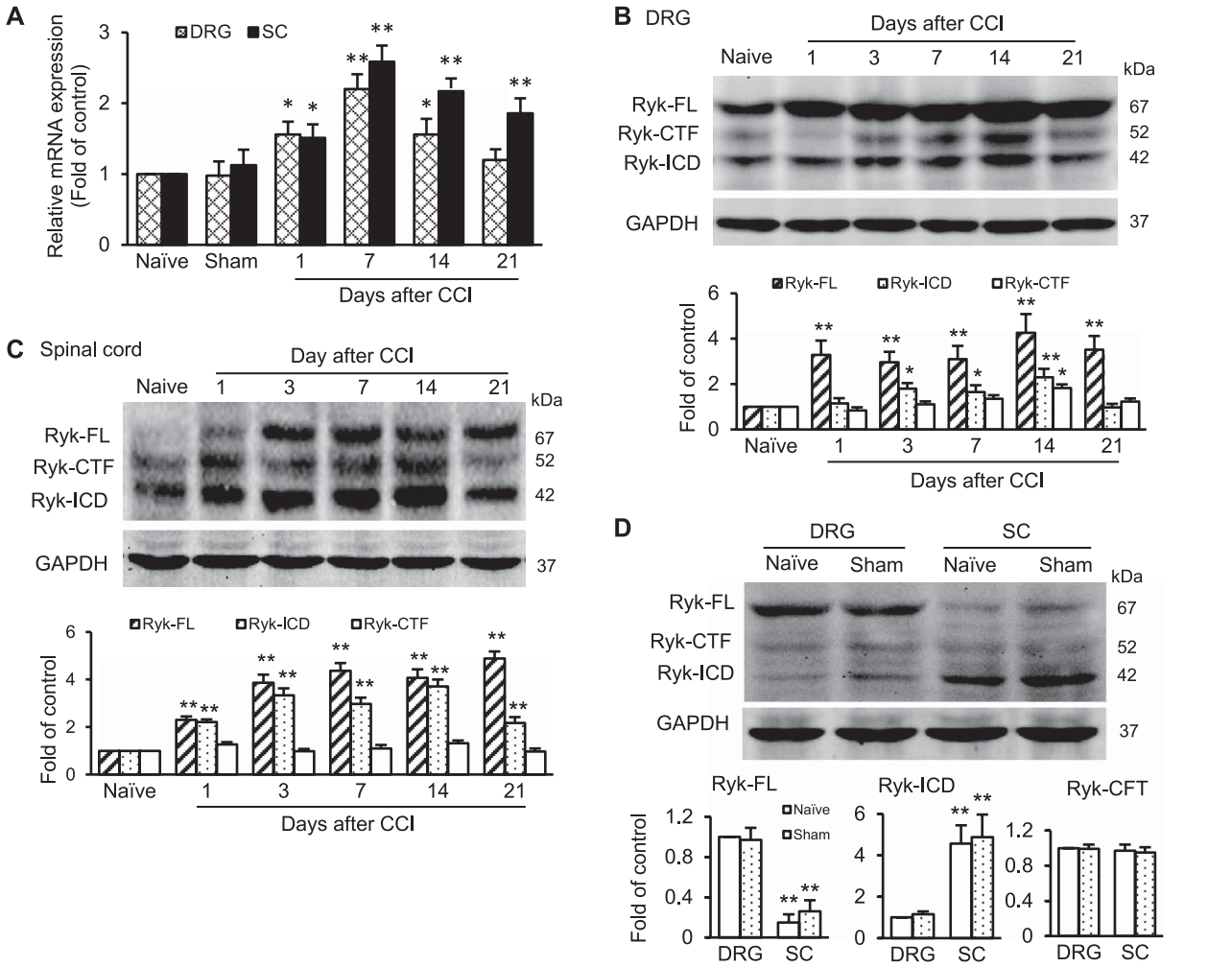

Figure 2. Expression of mRNA level and protein of Ryk receptor in dorsal root ganglion (DRG) and the spinal cord after chronic constriction injury (CCI). (A) Time courses of changes of level of mRNA of Ryk detected by quantitative real-time polymerase chain reaction. (B and C) Western blotting showing time course of expression of Ryk in DRG (B) and the spinal cord (C). (D) Western blotting showing different patterns of expression of the Ryk-FL and Ryk-ICD in DRG and the spinal cord. In (B–D) top: representative bands; bottom: data summary. Tissues in Sham group were collected on 7 days after surgery. Data are expressed as mean ± SEM (n = 4 in each group). *$P < 0.05$, **$P < 0.01$ vs Sham (A–C) or vs DRG naive (D) in the corresponding group (1-way analysis of variance). SC, spinal cord (D).

(0.03%) saline was inserted into the left carotid artery to monitor the blood pressure. The animal was then placed in a stereotaxic frame, paralyzed by an intravenous injection of pancuronium bromide (4 mg/kg), and artificially ventilated with air at a tidal volume of 15 mL/kg. Adequate anesthesia was confirmed intermittently during neuromuscular blockade in terms of the following 2 criteria: (1) the pupils were constricted and (2) the blood pressure remained stable during noxious stimulation. Core body temperature was monitored through a thermistor probe inserted into the rectum and maintained at 37.5 ± 0.5°C by means of a feedback-controlled heating pad under the ventral surface of the abdomen. In each experiment, phosphate-buffered saline was intermittently administered through a jugular vein cannula to maintain electrolyte balance. A laminectomy was performed to expose the lumbar spinal cord. The dura mater was then removed and the exposed cord was immediately covered with warm agar (2% in saline). After the agar hardened, a small hole was made above the recording site for application of drug or vehicle. The left common sciatic nerve was exposed at the mid-thigh level and covered with paraffin oil. At the end of each experiment, the animals were killed by an overdose of intravenous pentobarbital (200 mg/kg).

The afferent C-fibers–evoked field potential and its long-term potentiation (LTP) of synapses between the afferent C-fibers and the dorsal horn neurons were recorded on both naive and CCI rats. The protocol was similar to that previously described.[15,16,36] The amplifier Axoclamp 2B and DigiData 1322A and the PCLAMP-9 under Windows 2000 (Axon Instruments, Foster City, CA) were used for data acquisition and analysis. The signals were filtered (bandwidth: 0.1-500 Hz) and recorded at a sampling rate of 10 kHz. The recordings were made at depths of 150 to 400 μm from the surface of the spinal cord between L_4-L_5 with tungsten electrode (2 MΩ) (World Precision Instruments, Inc., Sarasota, FL). The sciatic nerve was stimulated by a bipolar platinum hook electrode. Single square pulses (0.5-millisecond duration) were delivered every 5 minutes to the sciatic nerve and were used as test stimuli. The strength of the test stimuli was 1.25× the minimum current that induced maximum C-fiber response. Long-term potentiation was induced by tetanic stimulation consisting of 100 electrical pulses, each 0.5 milliseconds, 100 Hz, at 2.5× the minimum current that induced maximum C-fiber, given in 4 trains or, when subthreshold, in 2 trains of 1-second duration at 10-second intervals to the sciatic nerve.

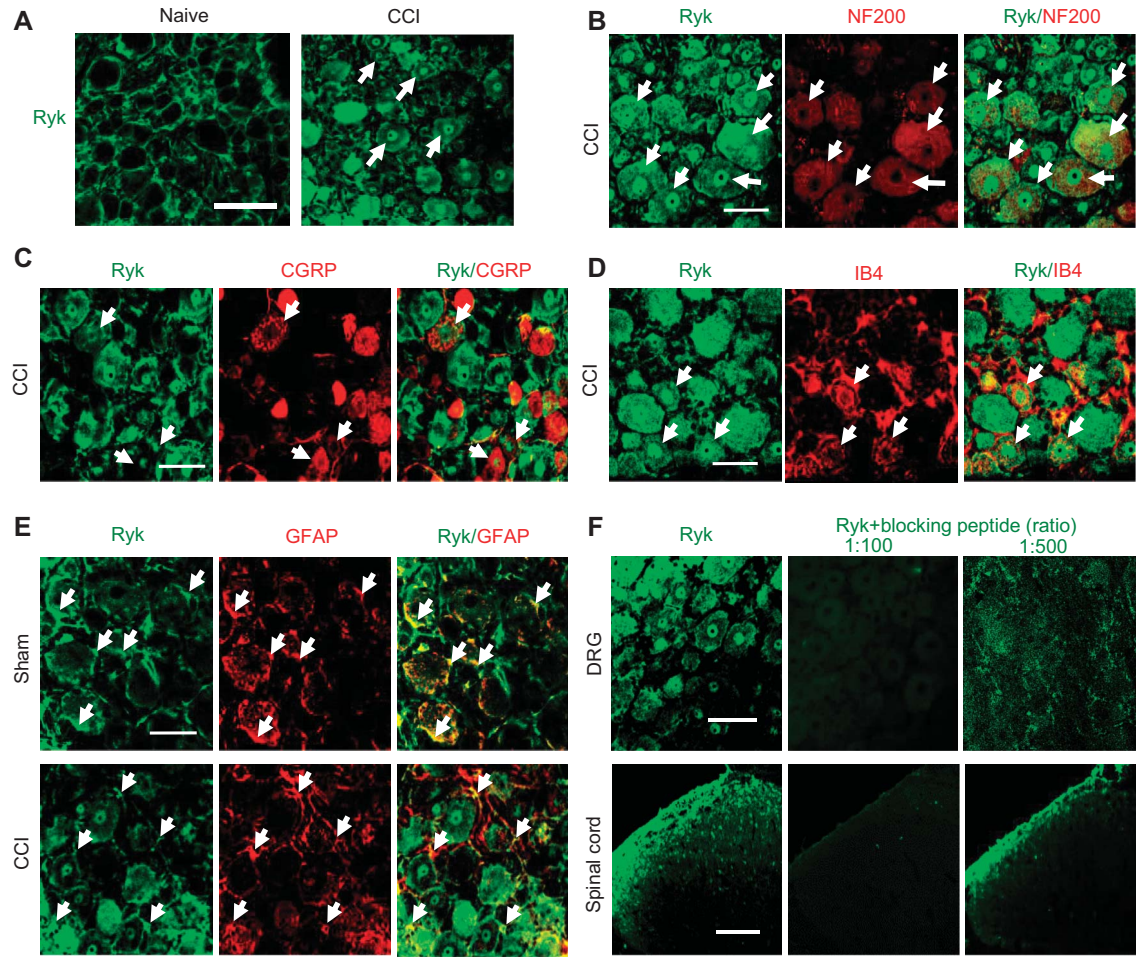

Figure 3. Cellular distributions of expression of Ryk protein in dorsal root ganglion (DRG) in Sham-operated and chronic constriction injury (CCI) rats. (A) Immunofluorescence showing expression and distribution of Ryk in naive and CCI-DRG. Note the great nucleus-transportation of Ryk in large-sized and medium-sized and small cells after CCI, but not in the naive DRG. (B–D) Cellular co-localization of the Ryk nucleus-transportation (green) in CCI-DRG with the large-sized neurons NF200 (B) and the small neurons identified as CGRP-positive (C) and IB4-positive (D) neurons. (E) Cellular co-localization of expression of Ryk with glia in naive- and CCI-DRG (GFAP, red). (F) Ryk antibody absorption control experiments in DRG and the spinal cord from CCI rats showing that the anti-Ryk antibody was highly specific. Concentrations and the ratio of Ryk and its blocking peptide: anti-Ryk, 0.02 μg/μL from 2 mg/mL, 1:100; blocking peptide, 0.01 μg/μL (1:100); and 0.002 μg/μL (1:500) from 1 mg/mL, respectively. Tissues were collected from naive (day 0) and CCI (day 7), respectively. Magnification: ×400. Bars: 100 μm (A), 50 μm (B–F).

2.11. Statistics

SPSS Rel 15 (SPSS Inc, Chicago, IL) was used to conduct all the statistical analysis. Alterations of expression of the mRNA and proteins detected and the behavioral responses to mechanical and thermal stimuli over time among groups were tested with 1-way and 2-way analysis of variance with repeated measures followed by Bonferroni post hoc tests, respectively. Individual *t*-tests were used to test specific hypotheses about differences between each operated or drug-treated group and its corresponding control group for each electrophysiological parameter tested and between the sham and CCI treatment for Western blot data. Chi-squared tests were used to identify differences in the incidence of effects. The nonparametric Wilcoxon signed-rank test and the Kruskal–Wallis test were used to test the C-fiber–evoked field potentials of dorsal horn neurons within and between the groups, respectively. All data are presented as mean ± SEM. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. CCI increases expression of Wnt3a and Wnt5b and activates Wnt/Ryk signaling in nociceptive pathways

Nerve injury-induced changes in DRG and the spinal cord are critical for generation of neuropathic pain. Wnt3a and Wnt5b are well characterized activators of the Wnt signaling pathways and are widely studied in the development and regeneration and also in the synaptic plasticity of the nervous systems.[4,9,17,24,33] We started with confirming the increased expression of Wnt3a, which was reported in our previous study,[35] and examining the possible changes of Wnt5b after CCI treatment. Our Western blot analysis showed that CCI produced a significant increase in the expression of both Wnt3a and Wnt5b in DRG and the spinal cord, respectively, in a similar pattern of rapid-onset (within 1 day) and long-lasting (at least 14 days in DRG and 21 days in the spinal cord). In DRG, Wnt3a and Wnt5b were peaked at 1-3 days and maintained at high levels from 1 to 14 days and recovered to control level at 21 days after CCI (**Figure 1A**). In the spinal cord, both Wnt3a and Wnt5b were maintained at high levels from 1 to 21 days, the last examination, after CCI (**Figure 1B**). As expected, sham-operation did not change the expression of Wnt3a and Wnt5b in DRG and the spinal cord (**Figure 1C**). These results indicate that both Wnt3a and Wnt5b may be initially increased in both DRG and spinal cord, whereas Wnt3a and Wnt5b in spinal cord may originate from spinal cord and be released from central terminals of DRG neurons.

Ryk receptor is an important Wnt target and can actively transduce the Wnt signal through Wnt-dependent nuclear translocation of the intracellular portion of Ryk.[7,25] Our results showed that CCI treatment significantly increased the mRNA level (**Figure 2A**) and protein expression of Ryk in DRG (**Figure 2B**) and the spinal cord (**Figure 2C**). The expression of Ryk included 3 separate parts, the full-length (Ryk-FL) localized in the membrane fraction, the C-terminal fraction (Ryk-CTF) localized exclusively in the cytoplasmic fraction, and the intracellular domain (Ryk-ICD). Chronic constriction injury produced a rapid-onset (within 1 days) and long-lasting (greater than 21 days) increase in the protein expression of Ryk-FL in DRG and the spinal cord and Ryk-ICD in

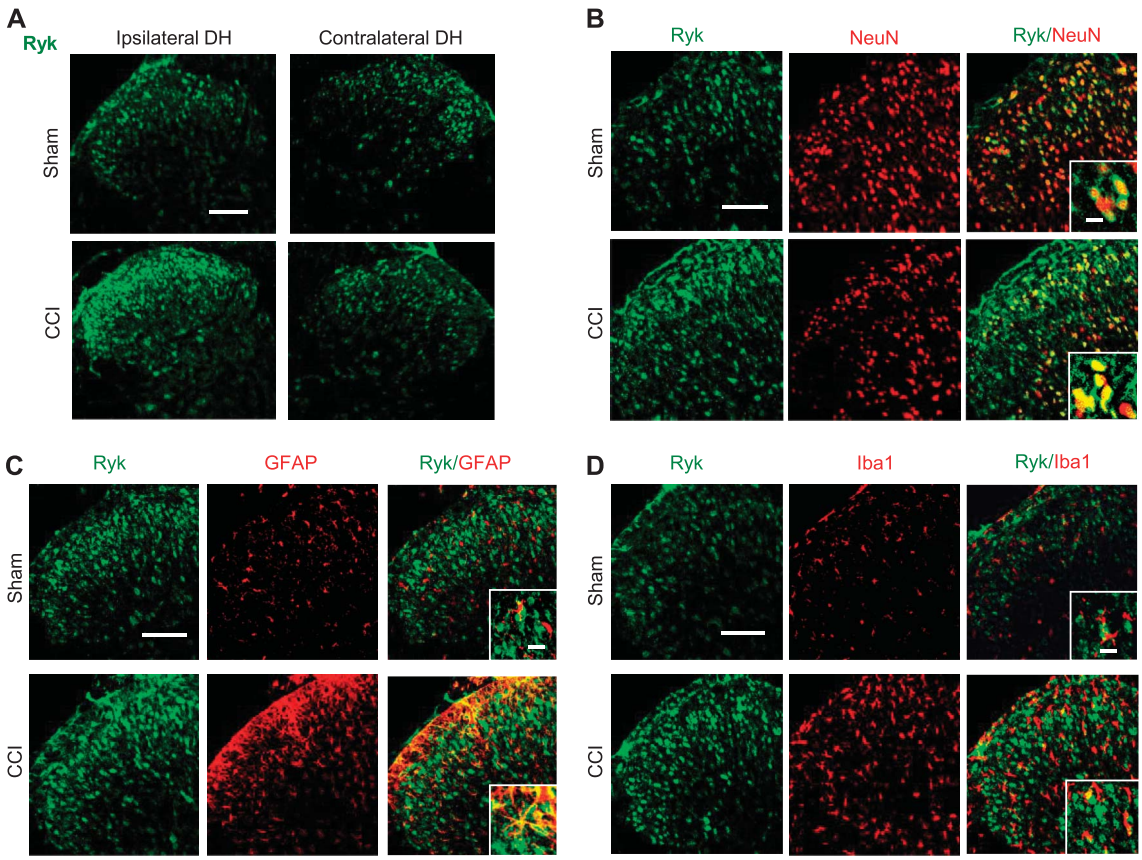

Figure 4. Expression and cellular distribution of Ryk protein in the spinal dorsal horn in Sham-operated and chronic constriction injury (CCI) rats. (A) Immunofluorescence showing distribution of Ryk expression. (B–D) Distribution and cellular co-localization of Ryk expression (green) in the superficial dorsal horn with NeuN-IR (NeuN, red) (B) and astrocytes (GFAP, red) (C), but basically not with microglial cells (Iba1 red) (D). Tissues were collected on 7 days after CCI or Sham. Magnification: ×200 (A), ×100 (B–D), ×400 (the inserts in B–D). Bars: 50 μm (A), 100 μm (B–D), and 10 μm (inserts in B–D). DH, dorsal horn of the spinal cord.

the spinal cord. Ryk-FL was peaked at 1 days in DRG and 3 days in spinal cord and was maintained at high level until 21 days, the last day observed, after CCI. Ryk-ICD was peaked in DRG at 3 days and in the spinal cord at 1 to 3 days, maintained at high level until 14 days in DRG and 21 days in the spinal cord, and recovered to the control level at 21 days in DRG. Ryk-CTF was seen a transient increase on the seventh day in DRG after CCI, but no change in the spinal cord (**Figure 2B, C**). We noted that, in the control animals (naive and sham-operated), expression of Ryk-FL in DRG was approximately 5 times more than that in the spinal cord, whereas expression of Ryk-ICD in the spinal cord was approximately 5 times more than that in the DRG. The Ryk-CTF in both DRG and the spinal cord was at similar level (**Figure 2D**).

Ryk expression, in naive DRG, was expressed in the neural membrane, but not in the neural plasma, and also in the neuron-surrounding glia (**Figure 3A**). Chronic constriction injury treatment greatly increased expression of Ryk in the neuronal plasma and resulted in tremendous nuclear transportation of Ryk (**Figure 3A–D**). Chronic constriction injury–induced increase of expression and nuclear transportation of Ryk were found distributed in all the large-sized and also in the CGRP- and IB4-positive small neurons identified by the neuronal markers NF200, CGRP, and IB4 (**Figure 3B–D**). Double immunostaining of Ryk with GFAP showed that Ryk was expressed in the satellite glia in naive and CCI DRG (**Figure 3E**). We performed the Ryk antibody absorption control experiments in DRG and the spinal cord and confirmed that the anti-Ryk antibody was highly specific (**Figure 3F**). In the spinal dorsal horn, the increased expression of Ryk was distributed in the superficial layers of the dorsal horn ipsilateral to CCI (**Figure 4A**) and co-localized predominantly with neuronal marker NeuN-IR (**Figure 4B**) or astrocyte marker GFAP (**Figure 4C**), but not observed consistently in microglial cells (**Figure 4D**). Expression of Ryk in the ventral horn was much lower than that in the dorsal horn and not changed after CCI (data not shown). These findings support that a rapid and long-lasting activation of the Wnt/Ryk signaling pathways occur after CCI.

3.2. Blocking Wnt/Ryk signaling suppresses the induction and persistence of neuropathic pain and accompanying neurochemical alterations after chronic constriction injury

Neuropathic pain is behaviorally characterized by the mechanical allodynia and thermal hyperalgesia in clinics. To determine the role of Wnt/Ryk signaling in neuropathic pain, we tested the possible analgesic effects of anti-Ryk antibody (anti-Ryk), functional-blocking Ryk receptor, on the induction and persistence of mechanical allodynia and thermal hyperalgesia in CCI-treated animals. With the goal of targeting the Wnt signaling in the DRG and in the spinal dorsal horn, the drugs were delivered by intrathecal administration (i.t., each 20 μL). In CCI-treated rats, repetitive administration of the anti-Ryk (4 μg) significantly delayed production of mechanical allodynia for 2 to 9 days (**Figure 5A**) and thermal hyperalgesia for 2 to 7 days (**Figure 5B**). A single treatment of the anti-Ryk (4 μg) 7 days after CCI produced a transient, significant inhibition of the established mechanical allodynia for 12 to 18 hours (**Figure 5C**) and thermal hyperalgesia for less than 12 hours (**Figure 5D**). The anti-Ryk did not significantly altered normal pain thresholds and locomotor activity (measured by the treadmill test using Exer-6M Treadmill, Columbus Ins., OH) in naive rats (data not shown) and resulted in any obvious side effects or animal death. These results demonstrate that inhibition of Wnt production and Wnt/Ryk signaling pathway can prevent and suppress the induction and persistence of CCI-induced behavioral hypersensitivity. These findings indicate an essential role of Wnt/Ryk signaling pathway in the development of neuropathic pain.

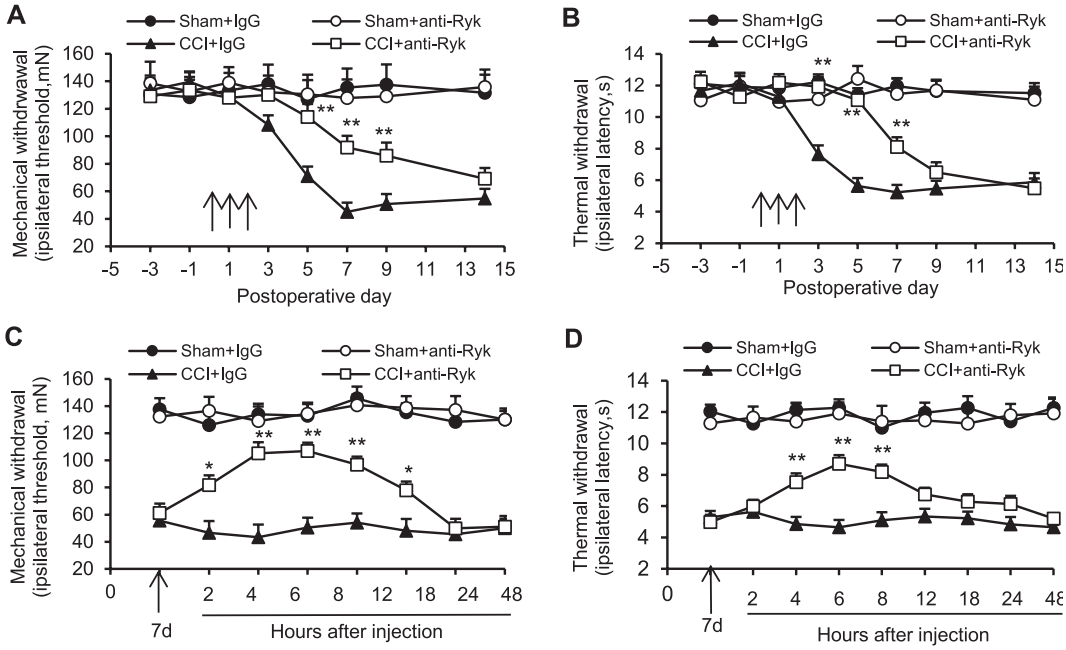

Figure 5. Activation of Wnt/Ryk signaling contributes to the development of neuropathic pain after chronic constriction injury (CCI) treatment in rats. (A and B) Effects of spinal administration of a Ryk antibody, anti-Ryk, on induction (A and B) and persistence (C and D) of mechanical allodynia and thermal hyperalgesia, respectively. Drug doses (i.t., 20 μL): anti-Ryk = 4 μg, IgG = 4 μg. IgG was used as control. Each administration is indicated by an arrow on the corresponding time point. Eight rats were included in each group and the surgery was performed on day 0. Two-way analysis of variance was used, and individual *t*-test was used to test the specific difference between the data point before drug administration and each of the data points after the drug administration. $^{**}P < 0.01$ vs CCI + IgG (A and B). $^{*}P < 0.05$, $^{**}P < 0.01$ vs the data point at day 7 before drug administration in the corresponding group (C and D).

3.3. Blocking activation of Ryk receptor inhibits increased intracellular Ca^{2+} activity, activation of NR2B receptor and the subsequent Ca^{2+}-dependent signals, and also hyperexcitability of dorsal root ganglion neurons after chronic constriction injury

Wnt can induce nuclear localization of intracellular domain of Ryk and Ryk is necessary for Wnt3a/5b-induced intracellular Ca^{2+} activity.[7,19,21] Given that activation of Wnt3a, Wnt5b and Ryk occur in DRG and the spinal dorsal horn after CCI, and that blockade of Ryk receptor functions attenuates CCI-induced hypersensitivity (**Figures 1–4**), we continued to examine the role of Ryk receptor expression in CCI-induced, pain-related, increased intracellular Ca^{2+} activity, activation of N-methyl-D-aspartate receptor (NMDAR), and the subsequent Ca^{2+}-dependent signals, and also the excitability of the DRG neurons. In vitro bath application of a Wnt agonist named Wnt agonist, an exogenous, selective activator of Wnt signaling, at 10, 20, and 40 μM, respectively, did not produce significant effect on the $[Ca^{2+}]_i$ in the small and medium-sized neurons in naive-DRG. However, in CCI-DRG, in vitro bath application of Wnt agonist at 20 μM significantly increased $[Ca^{2+}]_i$ in the small and medium-sized neurons. The Wnt agonist-induced increase of $[Ca^{2+}]_i$ in CCI-DRG was significantly reduced by the anti-Ryk (2 μg/mL), functional-blocking Ryk receptor. The anti-Ryk (0.5, 1.0, 2.0, and 4.0 μg/mL) produced a dose-dependent inhibitory effects on Wnt response. Representative responses and data summary are shown in **Figure 6A**.

Chronic constriction injury–induced DRG neuronal hyperexcitability manifested as decreased APCT and increased repetitive discharges.[30,37] In vitro bath application of anti-Ryk (2 μg/mL) significantly and greatly suppressed the increased neural excitability by reversing the decreased APCT and reducing the intracellular depolarizing current-induced repetitive discharges (**Figure 6B**). Furthermore, in vivo repetitive administration of anti-Ryk (4 μg, i.t., daily for 3 consecutive days on postoperative 7-9 days, respectively) suppressed CCI-induced activation (phosphorylation) of NMDAR subtype NR2B receptor and the subsequent Ca^{2+}-dependent signals CaMKII, Src/Tyr418, PKCγ, ERK, and CREB in the DRG (**Figure 7**).

These results demonstrate that activation of the Wnt/Ryk receptor signaling play critical roles in the increased activity of intracellular Ca^{2+}, activation of the NR2B and the subsequent Ca^{2+}-dependent signals, and also hyperexcitability of DRG neurons after CCI. These findings indicate an essential role of Wnt/Ryk signaling, through regulating the intracellular Ca^{2+} activity, in the development of the sensory neuron hypersensitivity after nerve injury.

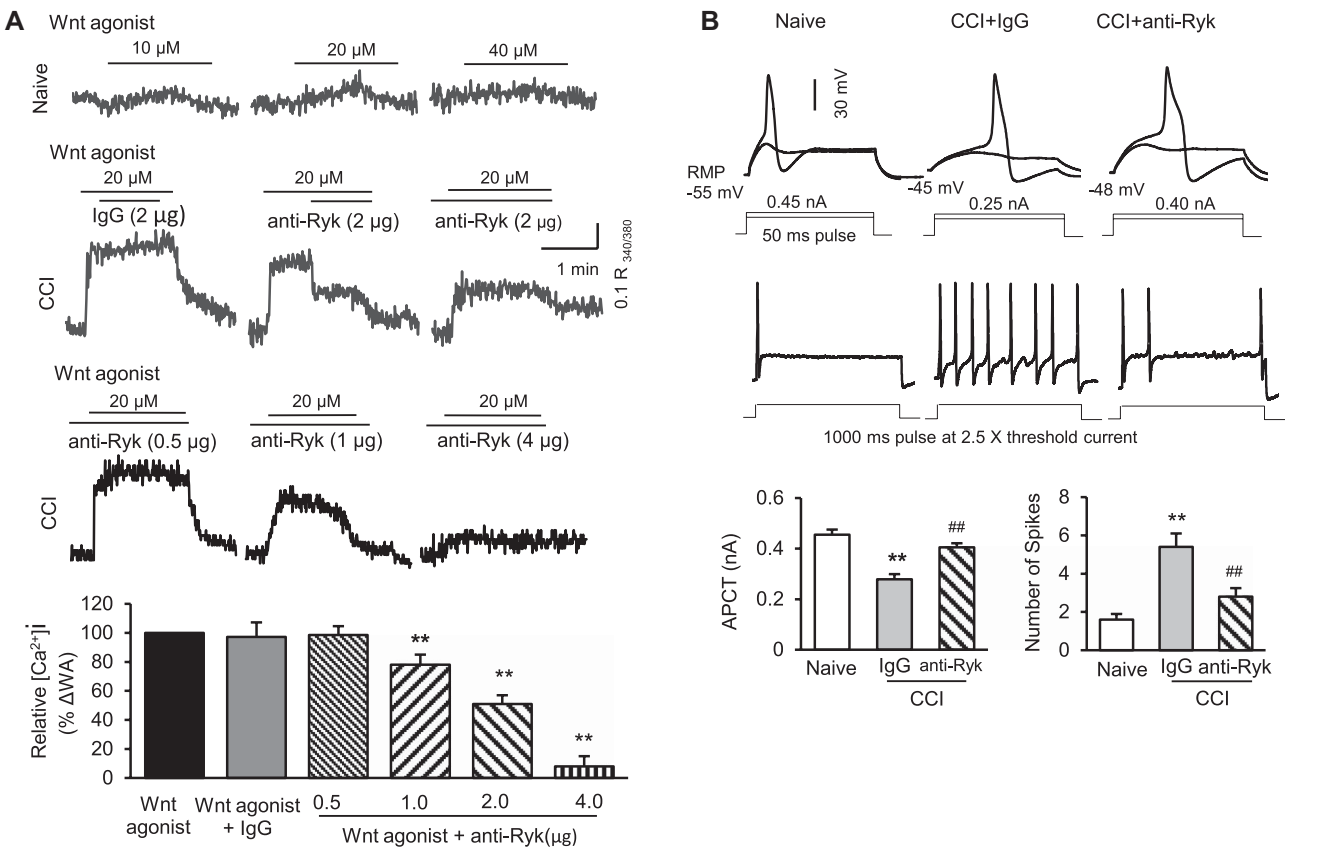

Figure 6. Activation of Wnt/Ryk receptor signaling contributes to increased activity of $[Ca^{2+}]_i$ and hyperexcitability of small dorsal root ganglion (DRG) neurons in chronic constriction injury (CCI)-DRG. (A) In vitro bath application of anti-Ryk (2 μg/mL) reduces the Wnt activator Wnt agonist (20 μM)-induced increase of activity of $[Ca^{2+}]_i$ in the DRG neurons (diameter: 30-45 μm) and the dose-dependent effects of anti-Ryk on Wnt response. Top traces: representative recordings of $[Ca^{2+}]_i$ from the naive control and CCI-treated, small and medium-sized DRG neurons. Bottom: data summary (histogram) showing dose-dependent effects of anti-Ryk on Wnt agonist-induced activity of $[Ca^{2+}]_i$ in CCI-DRG neurons. Number of neurons included in each of the groups: Wnt agonist = 19, Wnt agonist + IgG = 10, Wnt agonist + anti-Ryk (μg) = 15 (0.5), 18 (1.0), 42 (2.0), and 16 (4.0). Wnt agonist = 20 μM, IgG = 2 μg. $^*P < 0.05$, $^{**}P < 0.01$ vs Wnt agonist or Wnt agonist + IgG (1-way analysis of variance [ANOVA]). (B) Bath application of anti-Ryk (2 μg/mL) reverses the decreased APCT and reduces the repetitive discharges (number of spikes). Top: responses recorded with whole-cell patch electrodes under current clamp. Only 2 depolarizing 50-millisecond pulses (bottom) and responses (top) are illustrated in each case. Middle: repetitive discharge (spikes) patterns of neurons tested with 1-second pulses at 2.5 times the 50-millisecond APCT. Bottom: data summary. Number of DRG neurons in each group: naive = 17, CCI + IgG = 20, CCI + anti-Ryk = 18. $^{**}P < 0.01$ vs naive; $\#\#P < 0.01$ vs CCI + IgG (1-way ANOVA).

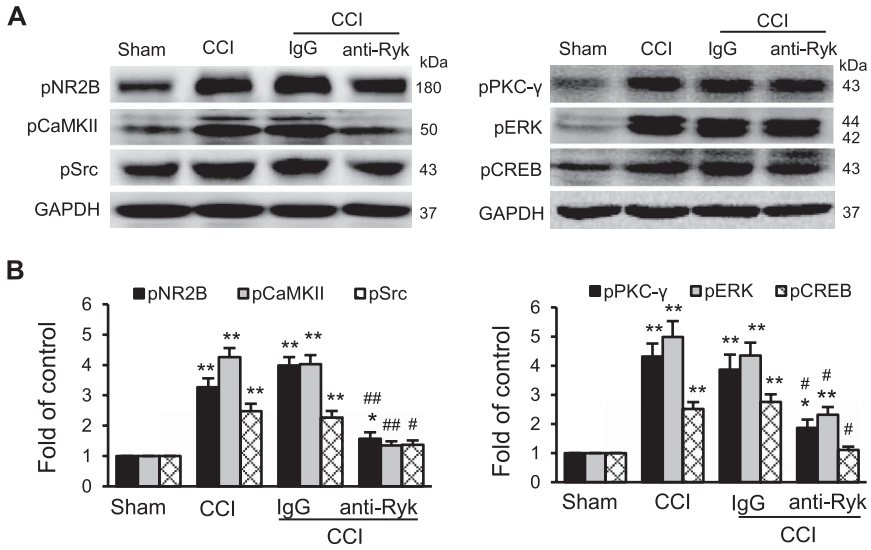

Figure 7. Activation of Wnt/Ryk receptor signaling contributes to activation of NR2B, CaMKII, Src, PKCγ, ERK, and CREB in chronic constriction injury–dorsal root ganglia (CCI-DRG). In vivo repetitive administration of anti-Ryk (4 μg, i.t.) inhibits phosphorylation of NR2B, CaMKII, Src, PKCγ, ERK, and CREB. Tissues were collected 4 hours after the last injection (n = 4 each group). (A) Representative Western blotting bands. (B) Data summary. *$P < 0.05$, **$P < 0.01$ vs Sham; #$P < 0.05$, ##$P < 0.01$ vs CCI or CCI + IgG (1-way analysis of variance).

3.4. Blocking activation of Ryk receptor inhibits N-methyl-D-aspartate receptor–dependent long-term potentiation of synapses between the afferent C-fibers and the dorsal horn neurons and activation of NR2B receptor and the subsequent Ca^{2+}-dependent signals after chronic constriction injury

The LTP of synapses between the afferent C-fibers and dorsal horn neurons may be associated with central sensitization contributing to painful consequences of nerve injury.[15] We examined roles of Ryk receptor in the development of the LTP. In sham-operated rats, in vivo topical i.t. anti-Ryk (1 μg in 100 μL) prevented the LTP induced by 4 high-frequency trains of nerve shock or by combination of 2 high-frequency trains, which alone did not evoke LTP, with NMDAR agonist NMDA (40 μM, 100 μL). The 4 high-frequency trains of nerve shock-induced LTP were also prevented by the NMDAR antagonist MK-801 (**Figure 8A, B**). In those rats 5 to 7 days after CCI treatment, 2 high-frequency trains alone, which did not evoke LTP in naive/sham-operated animals, induced LTP. This 2 high-frequency trains–induced LTP in CCI treated-rats was also blocked by pretreatment with anti-Ryk (1 μg in 100 μL) (**Figure 8C**). In addition, in vivo repetitive administration of anti-Ryk (4 μg, i.t., daily for 3 consecutive days) suppressed CCI-induced activation of NR2B and the subsequent Ca^{2+}-dependent signals CaMKII, Src/Tyr418, PKCγ, ERK, and CREB in the spinal cord (**Figure 9**).

These results support our hypothesis that activation of Wnt/Ryk receptor signaling may regulate plasticity of synapses between the nociceptive C-afferent fibers and the dorsal horn of the spinal cord neurons, probably through regulating the intracellular Ca^{2+} activity and the subsequent Ca^{2+}-dependent signals, after nerve injury, leading to the spinal cord central sensitization underlying the development of neuropathic pain.

4. Discussion

Our study reveals a critical role of Wnt/Ryk signaling in the induction and persistence of neuropathic pain after nerve injury. Nerve injury activates Wnt/Ryk signaling, which may contribute to neuropathic pain by regulating the excitability of DRG neurons and the synaptic plasticity of nociceptive dorsal horn neurons by modulating intracellular Ca^{2+} activity, activation of the NR2B receptor and the subsequent Ca^{2+}-dependent signals. The principle findings are 4-fold: (1) nerve injury (CCI treatment) causes a rapid-onset and long-lasting activation of the Wnt/Ryk signaling pathway in DRG neurons and glia and also in the spinal dorsal horn neurons and astrocytes; (2) spinal blocking of the Wnt/Ryk signaling pathways inhibits induction and persistence of CCI-induced neuropathic pain without affecting normal pain sensitivity and locomotor activity; (3) blocking activation of Ryk receptor inhibits CCI-induced hyperexcitability of the DRG neurons and the increased activity of intracellular Ca^{2+}, activation of the NR2B receptor, and the subsequent Ca^{2+}-dependent signals; (4) blocking activation of Ryk receptor inhibits CCI- or tetanic, electronic stimulation-induced enhanced plasticity of the synapses between nociceptive afferent C-fibers and the dorsal horn neurons and also activation of the NR2B receptor and subsequent Ca^{2+}-dependent signals. We know that DRG neural hyperexcitability and the dorsal horn neuron synaptic plasticity enhancement are essential to spinal central sensitization and the induction and persistence of neuropathic pain. These findings may support a new mechanism underlying neuropathic pain and a new therapeutic opportunity for its treatment after nerve injury.

Wnts are known to be important for various developmental processes. Studies have demonstrated dysregulation of Wnt signaling in certain diseases and disorders, eg, Wnt signaling is upregulated in schizophrenic brains[8] and downregulated in brain with Alzheimer disease.[3,22] Expression of many Wnt signal-related genes can be altered after cocaine exposure[23] or spinal cord contusion injury.[6] We have demonstrated that Wnt signaling may contribute to the development of neuropathic pain through the canonical, β-catenin–dependent signaling pathway, which induces production of proinflammatory cytokines including IL-18 and TNF-α in the spinal cord, and also through the noncanonical, β-catenin–independent pathway in DRG.[35] A study further indicates that Wnt signaling sensitizes DRG neurons by distinct noncanonical pathways, ie, the Wnt/PCP pathway acting through

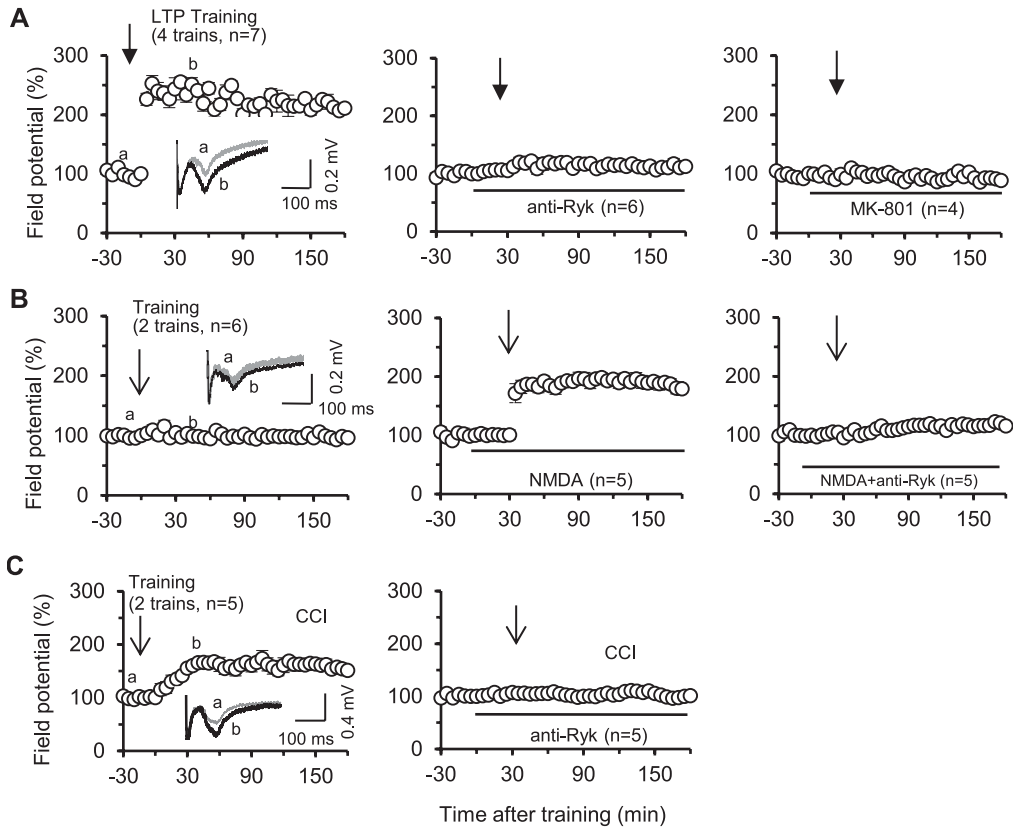

Figure 8. Activation of Wnt/Ryk receptor signaling contributes to the long-term potentiation (LTP) of synapses between the C-fibers and the dorsal horn neurons in vivo. (A–C) Topical spinal application of anti-Ryk (1 μg in 100 μL) significantly suppresses the different forms of LTP in sham (A and B) and chronic constriction injury (CCI) (C) animals, respectively. Amplitude of the field potentials was normalized to baseline values before LTP induction and plotted vs time (minute). Long-term potentiation training protocols in (A): 100 Hz, 2× threshold currents, 0.5 milliseconds, 100 pulses, 4 trains of 1-second duration at 10-second intervals. The subthreshold LTP training protocol used in (B and C) delivered only 2 trains. Examples of the C-fiber–evoked field potentials given in each figure were recorded before (a) and after (b) training (indicated by the arrow in each figure in A–C) or NMDA (B). The nonparametric Wilcoxon signed-rank test and the Kruskal–Wallis test were used to test the C-fiber–evoked field potentials of dorsal horn neurons within and between the groups, respectively. $P < 0.01$ in (A) left, (B) middle, and (C) left; $P > 0.05$ in the others in A–C.

Rac-1-JNK leads to rapid modulation of the molecular machinery involved in transducing mechanical pressure in peripheral sensory nerves, whereas Wnt-induced thermal hypersensitivity is largely mediated by the noncanonical calcium pathways acting through CaMKIIα and Src.[28] Here, we provide evidence that support a new idea that Wnt signaling may contribute to the development of neuropathic pain through a Ryk receptor-mediated pathway in the DRG and the dorsal horn. Peripheral nerve injury can cause dramatic and time-dependent changes in the physiological pattern of protein expression of Wnt3a, Wnt5b, and Ryk and also the mRNA level of Ryk in the DRG and the spinal cord. It is known that Wnt3a and/or Wnt5b can stimulate/induce the nuclear localization of the intracellular domain of Ryk, and that Ryk is necessary for Wnt-induced intracellular Ca^{2+} activity. Blocking activation of the Ryk receptor inhibits CCI-induced hyperexcitability of nociceptive DRG neurons. Furthermore, in the spinal cord, blocking activation of Ryk receptor can greatly suppress CCI- or tetanic stimulation-induced enhanced plasticity of synapses between the nociceptive afferent C-fibers and dorsal horn neurons. Finally, blocking Wnt/Ryk signaling in the spinal cord and DRG successfully suppresses the induction and persistence of neuropathic pain. We conclude that Ryk receptor-mediated Wnt signaling pathway is a new mechanism underlying neuropathic pain and a new therapeutic opportunity for its treatment after nerve injury. It is well known that the hyperexcitability of DRG neurons and enhanced synaptic plasticity of the dorsal horn neurons are essential and probably sufficient for spinal central sensitization, leading to neuropathic pain. Thus, we believe that the Wnt/Ryk signaling may contribute to neuropathic pain by regulating nociceptive primary sensory neuron excitability and nociceptive synaptic plasticity in the spinal cord.

Ryk is a single span transmembrane receptor with an intracellular tyrosine kinase domain and is a recently uncovered atypical receptor in Wnt signaling. Ryk family proteins have several common protein motifs and domains, the extracellular Wnt-binding WIF domain, a putative tetrabasic cleavage site, an intracellular domain bearing the receptor tyrosine kinase-related catalytic domain, and a carboxyterminal PDZ-binding domain. Ryk interacts with Wnt proteins through its extracellular WIF domain.[11,18,26] The intracellular domain of Ryk itself can function as the transducing molecule that brings extracellular signals from the cell surface into the nucleus.[7,19] In addition, Src is involved in Ryk signal transduction.[7] Our results have shown that expression of both extracellular and intracellular domains of Ryk are greatly increased after nerve injury. Blocking activation of Ryk in DRG and the spinal cord using an anti-Ryk antibody significantly suppresses the activation of the NR2B receptor, intracellular Ca^{2+} activity, and subsequent Ca^{2+}-dependent signaling proteins including Src, CaMKII, PKCγ, ERK, and

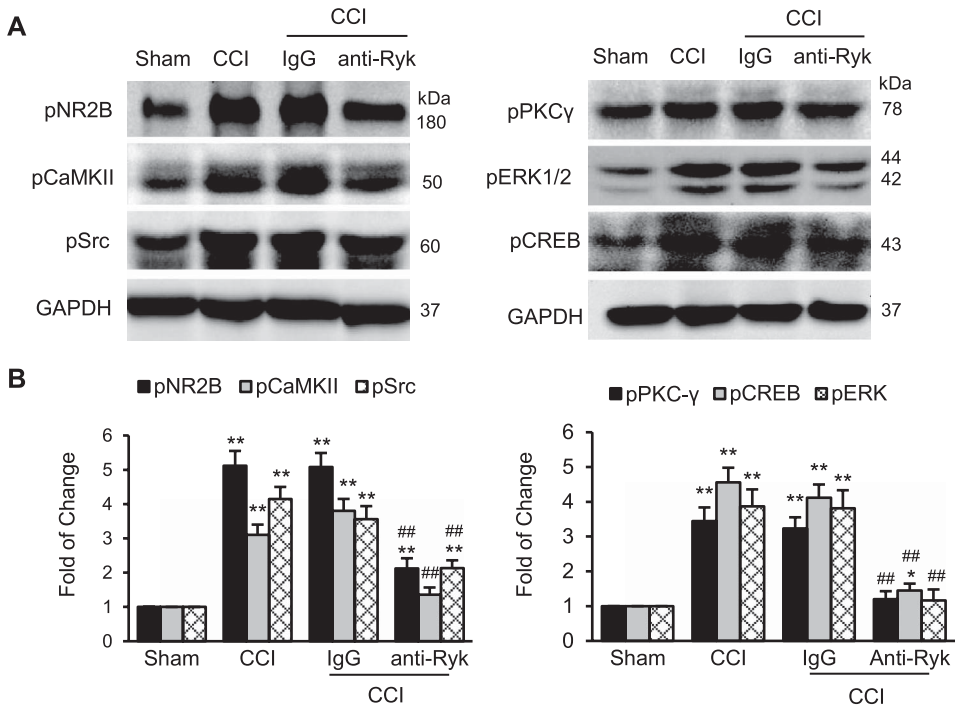

Figure 9. Repetitive spinal administration of anti-Ryk inhibits chronic constriction injury (CCI)-induced increased phosphorylation of NR2B, CaMKII, Src, PKCγ, ERK, and CREB in the spinal cord. Tissues were collected 4 hours after the last injection (n = 4 each group). (A) Representative bands of Western blotting. (B) Bottom: data summary. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Sham; $\#\#P < 0.01$ vs CCI or CCI + IgG (1-way analysis of variance).

CREB, and also the hyperexcitability of the DRG neurons and the enhanced synaptic plasticity of the dorsal horn neurons, after nerve injury. These findings show that activation of Ryk receptor may contribute to sensory neuron hyperexcitability and the enhanced synaptic plasticity of nociceptive dorsal horn neurons by regulating the NR2B receptor, intracellular Ca^{2+}

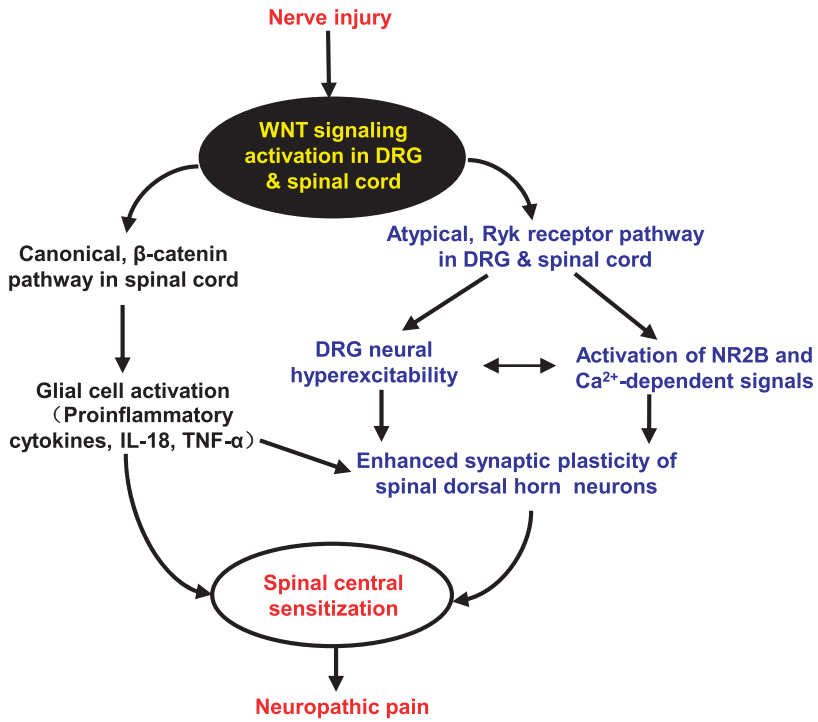

Figure 10. Schematic representation of the mechanisms of the Wnt signaling in dorsal root ganglion (DRG) and the spinal cord underlying neuropathic pain. The flowchart illustrates the possible pathways for Wnt signaling–induced spinal central sensitization and neuropathic pain. These pathways may include the atypical Ryk receptor pathway indicated in this study and the canonical, β-catenin–dependent pathway in the spinal cord and the noncanonical, β-catenin–independent pathway in DRG demonstrated in our most recent publication.[38] IL-18, interleukin-18; TNF-α, tumor necrosis factor-α.

activity, and subsequent Ca^{2+}-dependent signals including the Src-mediated pathways.

In addition, Ryk receptor may also function through the canonical Wnt signaling pathway in many different processes, such as mammalian cortex axon repulsion and axon outgrowth, which involve increased intracellular Ca^{2+} release. It is known that the Wnt/Ryk pathway may share at least 2 of the members, the Frizzled receptors and the Disheveled protein, and one of its targets, Tcf/Lef-regulated genes, with the canonical Wnt/Fz pathways.[7] We have demonstrated that the canonical, β-catenin–dependent Wnt signaling pathway in the spinal cord is critical to the development of neuropathic pain.[35] In this pathway, activation of Wnt/β-catenin is triggered by CCI-activated Wnts and Wnts binding frizzled receptors. This activation regulates the activity of proinflammatory IL-18 and TNF-α, which may directly contribute to the production and persistence of pain. Since Ryk may share the frizzled and Disheveled pathways of Wnt signaling, activation of Ryk may also act through the canonical Wnt/frizzled/β-catenin pathway to activate the proinflammatory cytokines IL-18 and TNF-α in the spinal cord and thus contribute to neuropathic pain. Mechanisms underlying contributions of Wnt signaling to neuropathic pain are summarized in **Figure 10**.

Clinical approaches for treating neuropathic pain are very limited and the specific cellular and molecular mechanisms underlying neuropathic pain remain elusive. Our findings in this study and the recent report[35] have demonstrated that Wnt signaling, which plays essential roles in various processes during development of nervous systems and is activated after nerve injury, is critical to the induction and persistence of neuropathic pain. This indicates a critical mechanism underlying the pathogenesis of neuropathic pain. In the spinal cord, Wnt signaling may act through the canonical, β-catenin–dependent signaling pathway and the atypical Ryk pathway. In the DRG, Wnt signaling may act through the noncanonical, β-catenin–independent pathway and the atypical Ryk pathway. In addition, Wnt signaling can sensitize DRG neurons through distinct noncanonical pathways.[28] Wnt/Ryk signaling, as shown in this study, may contribute neuropathic pain by sensitizing DRG neurons and enhancing the nociceptive synaptic plasticity in the dorsal horn neurons by regulating the activity of the NR2B receptor and intracellular Ca^{2+}, and also subsequent Ca^{2+}-dependent signals. These findings suggest that targeting the Wnt signaling pathways may be an effective approach for treating neuropathic pain.

Interestingly, recent studies have shown that injury-induced Wnt/Ryk pathway activation inhibits axon regeneration and thus may contribute to poor postinjury axon regeneration.[7,17,20] This suggests that inhibiting nerve injury-induced Wnt/Ryk pathway activation may attenuate neuropathic pain and simultaneously stimulate axon regeneration.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgements

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